



## Revista Brasileira de Hematologia e Hemoterapia Brazilian Journal of Hematology and Hemotherapy

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### Original article

# Impact of a confirmatory RhD test on the correct serologic typing of blood donors



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#### ARTICLE INFO

##### Article history:

Received 27 February 2015

Accepted 3 June 2015

Available online 9 July 2015

##### Keywords:

Blood group

Molecular genetics transfusion

Red blood cell antigens

#### ABSTRACT

**Background:** The RHD gene is highly polymorphic, which results in a large number of RhD variant phenotypes. Discrepancies in RhD typing are still a problem in blood banks and increase the risk of alloimmunization. In this study, the RhD typing strategy at a blood bank in Brazil was evaluated.

**Methods:** One-hundred and fifty-two samples typed as RhD negative and C or E positive by routine tests (automated system and indirect antiglobulin test using the tube technique) were reevaluated for RhD status by three methods. The method with the best performance was implemented and evaluated for a period of one year ( $n=4897$  samples). Samples that were D positive exclusively in the confirmatory test were submitted to molecular analysis.

**Results:** The gel test for indirect antiglobulin testing with anti-D immunoglobulin G (clone ESD1) presented the best results. Seventy samples (1.43%) previously typed as RhD negative showed reactivity in the gel test for indirect antiglobulin testing and were reclassified as D positive. D variants that may cause alloimmunization, such as weak D type 2 and partial D<sup>VI</sup>, were detected.

**Conclusion:** The confirmatory RhD test using the gel test for indirect antiglobulin testing represents a breakthrough in transfusion safety in this blood center. Our results emphasize the importance of assessing the blood group typing strategy in blood banks.

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## Introduction

The D blood group antigen is the most clinically important protein of the Rh system due to its involvement in hemolytic

transfusion reactions and hemolytic diseases of the fetus and newborns.<sup>1</sup> Despite methodological advances, discrepancies in Rh typing are still a problem during routine immunohematology service tests.<sup>2-4</sup>

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<http://dx.doi.org/10.1016/j.bjhh.2015.06.001>

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The RHD gene is highly polymorphic as it has more than 200 alleles. This results in a large number of RhD variant phenotypes.<sup>5</sup> Rh discrepancies may arise when an individual has a variant of the D antigen, such as partial D or weak D, and may be mistyped as D negative. These discrepancies can cause incorrect blood component transfusions, leading to increased risk of alloimmunization.<sup>3</sup>

In addition to the great number of variants, a major cause of discrepancies in RhD typing is the existence of several RhD typing methods and reagents with different sensitivities.<sup>6-9</sup> Blood group typing strategies and policies for the selection of methods and reagents vary between countries.<sup>2</sup> In addition, few centers in the world routinely perform molecular tests for the Rh typing of blood donors. Therefore, despite the best efforts, the number of D variant blood donors typed as D negative, even by an indirect antiglobulin test (IAT), is greater than previously expected.<sup>10</sup> This misclassification results in an increased risk of anti-D alloimmunization, which has important health implications, especially for women of childbearing age.

In this study, the effectiveness of serological tests used for the RhD typing of blood donors were evaluated in a large blood center in Brazil (Fundação Hemominas).

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## Methods

### Study population

In the first step of this study, red blood cell (RBC) samples from 152 blood donors phenotyped as D negative who were C or E positive in routine tests using an automated system (Olympus PK72000) were analyzed. These samples were tested for weak D by three different serological methods and using different reagents.

The second step consisted of implementing and evaluating a protocol of RhD phenotyping based on an indirect antiglobulin technique using LISS/Coombs gel cards (Diamed, Switzerland) and a monoclonal reagent, anti-D immunoglobulin G (IgG) (clone ESD1). This confirmatory test was evaluated and compared to the IAT tube test over one year to verify the RhD status of blood donors ( $n=4897$ ) who were phenotyped as D negative and C or E positive or with extended phenotyping.

The last step of this study consisted of using molecular methods to confirm the RhD status of the samples that were typed as D positive exclusively in the confirmatory gel test for indirect antiglobulin testing ( $n=39$ ).

### Serological studies

RhD typing was first performed with an automated system (Olympus PK 7200) using two anti-D reagents: a monoclonal blend of anti-D clones (MS 201 and MS 26; Fresenius Kabi, Brazil) diluted 1-32 and 1-64 in saline solution, and a monoclonal antibody blend of RUM-1 and MS-26 clones (Lorne Reagents) diluted 1-64 in saline solution. All donor RBCs were treated with 0.2% bromelain. RBCs found to be D negative were further tested for weak D by IAT (tube technique) using a monoclonal anti-D blend (Fresenius Kabi, Brazil).

All D negative RBC samples were tested with monoclonal antisera (anti-CDE), and clones P3X61, P3X25513G8 and P3X234 (Fresenius Kabi, Brazil) in a tube, according to the manufacturer's instructions.

The samples typed as D negative and C or E positive, as described above, were tested for weak D (IAT) using three protocols: anti-D blend clones MS-26 and TH-28 (Diamed, Switzerland) in a tube; anti-D blend clones MS-26 and TH-28 in gel cards; and the anti-D IgG clone ESD1 in gel cards. The gel tests were performed on cards (ID LISS Coombs; Diamed, Switzerland) and were incubated at 37 °C for 15 min.

The protocol for weak D using anti-D ESD1 in gel cards was implemented in the blood center as a routine confirmatory test and was evaluated for one year.

### Molecular analyses

Samples typed as D positive in the confirmatory test were submitted to multiplex polymerase chain reaction (PCR) to amplify five RHD-specific exons: 3, 4, 5, 7 and 9.<sup>11,12</sup> The samples were also analyzed for the presence of weak D Types 1, 2 and 3 using a PCR-sequence specific primer (PCR-SSP)<sup>13</sup> and were tested for the presence of other D variants using RHD BeadChip™ analysis (BioArray Solutions, Immucor) according to the manufacturer's instructions.

### Ethical considerations

The Ethics Committee of the Fundação Hemominas approved the study (CEP no.136.163).

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## Results

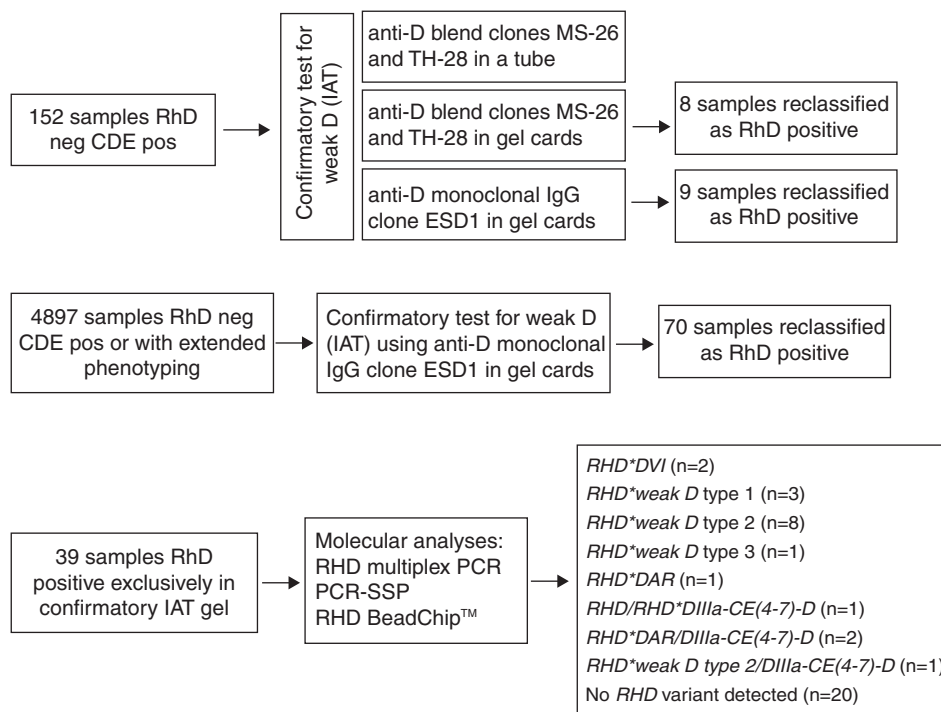
Samples from 152 blood donors typed as D negative and C or E positive by an automated system were evaluated using the three protocols. Nine (5.9%) of the samples phenotyped as D negative by the tube test presented positive results in gel cards (LISS/Coombs) using anti-D IgG (clone ESD1). The anti-D blend reagent (clones MS-26 and TH-28) detected positivity of eight of these samples. The D positive status of the nine samples was confirmed by RHD genotyping.

Based on these results, Fundação Hemominas decided to implement a protocol to confirm RhD typing using anti-D IgG monoclonal (clone ESD1) in gel cards of all blood donors phenotyped as D negative and C or E positive or with extended phenotyping.

During one year, 4897 samples of blood donors from different regions of Minas Gerais (Brazil) were referred to this blood center to confirm the RhD typing. All RBC samples had originally been tested with an anti-D blend (clones MS-26 and TH-28; Diamed-Biorad) by the tube test.

The results of this study showed that 70 samples (1.43%) previously typed as RhD negative by the tube test presented weak reactivity in gel cards using anti-D IgG (clone ESD1) and were reclassified as D positive.

In the subsequent step of this study, RHD genotyping was performed of 39 samples typed as D negative by the tube test that were weakly positive in the confirmatory gel test.



**Figure 1 – Flowchart for the serological and molecular confirmatory RhD tests performed for blood donors.**

Twenty-seven of these 39 samples amplified all of the *RHD*-specific exons by multiplex PCR, and twelve samples failed to amplify at least one *RHD* exon. The samples were further assessed by PCR-SSP and RHD BeadChip™ analysis and were characterized as follows: *RHD*\*DVI ( $n=2$ ), *RHD*\*weak D type 1 ( $n=3$ ), *RHD*\*weak D type 2 ( $n=8$ ), *RHD*\*weak D type 3 ( $n=1$ ), *RHD*\*DAR ( $n=1$ ), heterozygous *RHD*/*RHD*\*DIIIa-CE(4-7)-D ( $n=1$ ), *RHD*\*DAR/DIIIa-CE(4-7)-D ( $n=2$ ) and *RHD*\*weak D type 2/DIIIa-CE(4-7)-D ( $n=1$ ). In twenty samples, *RHD* variants were not detected by RHD BeadChip analysis.

Figure 1 summarizes the results.

## Discussion

The correct determination of the RhD phenotype is of great importance in transfusion medicine. The existence of a large number of polymorphisms of the *RHD* gene and the plethora of variant RhD phenotypes, coupled with the availability of a large number of methods and reagents, makes RhD phenotyping a challenge.<sup>3</sup>

To evaluate RhD phenotyping in Fundação Hemominas, three different methods were compared to confirm weak D tests (IAT) of samples from 152 Brazilian blood donors. Gel cards presented the best results to detect anti-D IgG (clone ESD1); this technique could be used as a confirmatory test. This method was implemented in the blood Center and evaluated over the course of one year with 4897 blood donors. During this period, 1.43% ( $n=70$ ) of the samples previously typed as D negative by the tube test presented positive results exclusively in the gel test for indirect antiglobulin testing, and were reclassified as D positive.

The molecular tests used to confirm the RhD status of 39 samples typed as D negative by IAT and as weak D positive in the confirmatory gel test revealed the presence of D variants [*RHD*\*DVI, *RHD*\*weak D type 1, *RHD*\*weak D type 2, *RHD*\*weak D type 3, *RHD*\*DAR, the heterozygous *RHD*/*RHD*\*DIIIa-CE(4-7)-D, *RHD*\*DAR/DIIIa-CE(4-7)-D and *RHD*\*weak D type 2/DIIIa-CE(4-7)-D] in 19 samples (48.7%). Weak D type 2 is among the most prevalent D variants in Brazil.<sup>9</sup> While weak D type 2 presents a relatively low antigenic density, it is expected to react in a tube or in gel without an antiglobulin test.<sup>14</sup> However, some studies show that weak D type 2 and D<sup>VI</sup>, as well as some other D variants, may not react with some anti-D reagents in a direct test and may react very weakly in the tube test.<sup>9</sup> Therefore, these samples may occasionally be mistyped as D negative in routine tests.<sup>4,9,15</sup>

There are many factors that can affect the reproducibility and reliability of the tube test. These factors include problems in the washing step, variability in individual reading techniques and the level of expertise needed to accurately grade results.<sup>16</sup> All of these factors, coupled with the variable reactivity displayed by some anti-D reagents with D variant samples and the heterogeneous ethnic composition of Brazilian blood donors which can generate a complex scenario of RhD variants, may explain the results obtained herein.<sup>9,17</sup>

Interestingly, no RhD variant was detected by RHD BeadChip for twenty of the 39 samples (51.3%). However, the amplification of all of the *RHD* exons evaluated and the positivity of the confirmatory serologic tests indicate that these are RhD positive samples. This result can be explained by the fact that the RHD BeadChip kit detects approximately 80 variants while there are currently hundreds of known RhD variants. Moreover, a recent study described a high prevalence of the

RHD gene in Brazilian samples phenotyped as C or E positive with a very low density of the D antigen, mainly weak D type 38, and indicates the need for the implementation of RHD molecular screening in serologically D negative and C or E positive Brazilian donors to reduce the risk of D immunization associated with the erroneous transfusion of weak D RBCs to D negative recipients.<sup>18</sup>

## Conclusion

The implementation of the confirmatory RhD test using the gel test for indirect antiglobulin testing for RhD negative and CDE positive samples in the blood center represents a breakthrough in transfusion safety thereby allowing the correct RhD typing of dozens of samples that would have been erroneously classified as D negative by direct automated RhD phenotyping followed by the tube test. Moreover, this strategy demonstrates the technical feasibility, even in a large blood center such as the Fundação Hemominas, which types 270,000 samples for ABO/RhD and approximately 37,700 IAT tests for weak D annually.

These results emphasize the importance of assessing the blood group typing strategy in blood banks. This evaluation is important because RhD typing discrepancies can occur even with the use of standard techniques and reagents that the manufacturers claim are effective for detecting weak D variants. Thus, each center must evaluate which protocol is best suited to local conditions. In addition, RHD genotyping again proved to be a very useful tool in identifying inconclusive RhD typing cases.

## Funding

FAPEMIG, Fundação Hemominas, FAPESP.

## Conflicts of interest

The authors have no conflicts of interest to disclose.

## Acknowledgments

This work was supported by Fundação Hemominas; Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), grants no. APQ-00485-12, BIP-00064-14, BIP-00009-14; and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grant no. 2012/50927-0.

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